

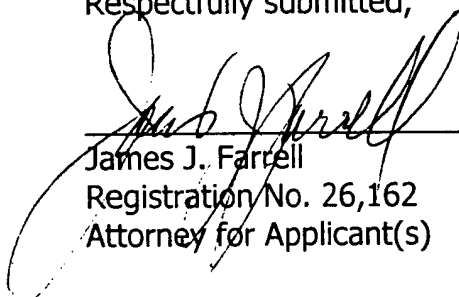
REMARKS

The specification and claims have been amended to insert the Sequence I.D. numbers in the appropriate place. In addition, a written copy of the sequence listing and a computer readable copy have been included together with the instant amendment.

Applicants' attorney has been informed and believes that the content of the sequence disk copy and the paper copy are identical and include no new matter.

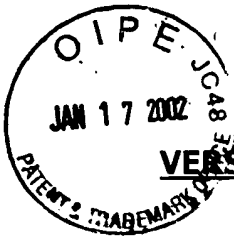
In view of the foregoing amendments and comments, early favorable action is solicited.

Respectfully submitted,



James J. Farrell
Registration No. 26,162
Attorney for Applicant(s)

JJF/lac
(201) 840-2437



VERSION WITH MARKINGS TO SHOW CHANGES MADE

In The Specification:

Please amend the specification as follows:

Page 5, line 29 and ff:

A second aspect of the invention relates to an AFP which can be derived from grass, said AFP having an amino acid sequence (Seq. ID No. 1) from the N-terminus of:

Page 7, line 21 and ff:

Applicants have also determined the nucleic acid sequence that encodes the above described AFP. Accordingly a second aspect of the invention relates to a nucleic acid sequence capable of encoding for the AFPs of the invention. Preferably said nucleic acid has the sequence (Seq. ID No. 2) of:

Page 21, line 1 and ff:

tolerant RI active protein from *Lolium perenne* (Seq. ID No. 3) and the 27 kDa protein from *Poa pratensis* (Seq. ID No. 4)

Lolium - D E Q P N T I S G X N N T V R X G

Poa - A E T P N T I S G T N N

Page 24, line 8 and ff:

A degenerate oligonucleotide primer (Lol 1) was designed and synthesised from the protein N-terminus sequence (ASP-GLU-GLN-PRO-ASN-THR-ILE) of Seq. ID No. 4, as GAYGARCARCCIAAYACIAT where Y = C+T, R = A+G and I = Inosine. (Seq. ID No. 5)

Page 24, line 13 and ff:

First strand cDNA was prepared from 5µg of 30 day cold acclimated *Lolium perenne* leaf RNA using Superscript Reverse Transcriptase (Stratgene) and an oligonucleotide primer OG1

(GAGAGAGGATCCTCGAG (T) 15) (Seq. ID No. 6) according to the manufacturers instructions. 1% of the first strand cDNA was used as a template, together with Lol1 and OG1 primers, in PCR reactions. The reactions were carried out in a thermal cycler using Taq DNA Polymerase (Gibco BRL) for 30 cycles (1minute at 94°C, 1 minute at 55°C and 1 minute at 72°C) after an initial denaturation step of 2 minutes at 94°C.

Page 24, line 24 and ff:

A PCR product of ~ 600 bp was amplified and subsequently purified from a 1% agarose gel and cloned into the pTAg vector (R&D systems) according to the manufacturers instructions. The cloned PCR product was sequenced on a Perkin Elmer (Applied Biosystems) automated DNA sequencer using T3 and T7 primers. It contained an open reading frame substantially similar to Seq. ID No. 2:

Page 25, line 8 and ff:

and the deduced amino acid sequence was substantially similar to Seq. ID No. 1:

Page 26, line 16 to end of page and Page 27, line 1 to line 7:

The Lolium cDNA was cloned into the pPIC9 vector as a PCR amplification fragment, with compatible restriction ends for ligation into the pPIC9 vector. This was produced using Lolium cDNA as the template and the primers GTATCTCTCGAGAAAAGAGATGAGCAGCCGAACACGATT (Seq. ID No. 7) and TTAATTCGCGCCGCTGTAGGAAAAGTATGGTATATC (Seq. ID No. 8) which introduced a Xho1 restriction site at the 5' end and a Not1 restriction site at the 3' end of the amplification fragment and ensured that the Lolium cDNA was in frame with the secretion signal open reading frame. The reactions were carried out in a thermal cycler using Taq DNA polymerase and Pfu proof reading enzyme (Boehringer Mannheim) for 30 cycles (1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C). All subsequent PCR reactions were carried out under the same conditions but without Pfu enzyme. The Xho1/Not1 cDNA fragment was then cloned into the Xho1/Not1 digested pPIC 9 vector and transformed into competent E Coli cells (strain XL1-blue). After transformation, they were plated onto LB plates with 50µg ml ampicillin and grown at 37°C for 16 hours. Then, 20 ampicillin resistant transformants were picked and

analysed for integration of the Lolium cDNA by PCR using the 5' AOX1 and the 3' AOX1 primers that had been synthesised as specified in the Invitrogen Kit Manual.

Page 33, line 15 to end and Page 34, line 1:

The Lolium cDNA was cloned into the pET-32 plasmid as a PCR amplification fragment, with compatible restriction ends for ligation into the multiple cloning site of the pET-32 plasmid. This was produced using Lolium cDNA as the template and a 5' oligonucleotide primer (TTTGCGCTCGAGTTAAGCGTCTGTCACGACTTTG) (Seq. ID No. 9) and 3' oligonucleotide primer (TTTGCGCCATGGATGAACAGCCGAATACGATTTC) (Seq. ID No. 10). This introduced a NcoI restriction site at the 5' end and a XhoI restriction site at the 3' end of the amplification fragment. The reactions were carried out in a thermal cycler using Taq DNA polymerase for 25 cycles (denaturation at 94°C, annealing from 35°C to 60°C, extension at 72°C). All subsequent PCR reactions were carried out under the same conditions. The NcoI/XhoI PCR amplified fragment was then cloned into the NcoI/XhoI digested pET-32 plasmid and transformed into competent non-expression *E.Coli* cells (strain XL1-Blue). After transformation, they were plated onto LB-ampicillin plates and grown at 37°C for 16 hours. 10 ampicillin resistant transformants were picked and analysed for integration of the Lolium cDNA by PCR using the pET upstream and downstream primers from Novagen.

In The Claims:

Please amend the claims as follows:

3. (Amended) Anti-freeze protein according to claim 1 having at least 80% overlap with the following amino acid sequence (Seq. ID No. 1):

claim 3

D-E-Q-P-N-T-I-S-G-S-N-N-T-V-R-S-G-S-K-N-V-L-A-G-N-D-N-T-V-I-S-G-D-N-N-S-V-S-G-S-N-N-T-V-V-S-G-N-D-N-T-V-T-G-S-N-H-V-V-S-G-T-N-H-I-V-T-D-N-N-N-N-V-S-G-N-D-N-N-V-S-G-S-F-H-T-V-S-G-G-H-N-T-V-S-G-S-N-N-T-V-S-G-S-N-H-V-V-S-G-S-N-K-V-V-T-D-A

as well as modified versions thereof.

8. (Amended) Nucleic acid sequence of claim 7, having the sequence (Seq. ID No. 2):

GAT GAA CAG CCG AAT ACG ATT TCT GGG AGC AAC AAT ACT GTC AGA TCC GGG AGC
AAA AAT GTT CTT GCT GGG AAT GAC AAC ACC GTC ATA TCT GGG GAC AAC AAT AGT
GTG TCT GGG AGC AAC AAC ACT GTC GTA AGT GGG AAT GAC AAT ACC GTA ACC GGC
AGC AAC CAT GTC GTA TCA GGG ACA AAC CAT ATC GTT ACA GAC AAC AAC AAT AAC
GTA TCC GGG AAC GAT AAT AAT GTA TCC GGG AGC TTT CAT ACC GTA TCC GGG GGG
CAC AAT ACT GTG TCC GGG AGC AAC AAT ACC GTA TCT GGG AGC AAC CAC GTT GTA
TCT GGA AGC AAC AAA GTC GTG ACA GAC GCT TAA

and alleles thereof.

Please enter an Abstract as follows:

Abstract

Anti-freeze protein comprising at least 40% of the amino acids S,T or N and having at least 80% overlap with the following amino acid sequence (Seq. ID No. 1):

D-E-Q-P-N-T-I-S-G-S-N-N-T-V-R-S-G-S-K-N-V-L-A-G-N-D-N-T-V-I-S-G-D-N-N-S-V-S-G-S-N-N-T-V-
V-S-G-N-D-N-T-V-T-G-S-N-H-V-V-S-G-T-N-H-I-V-T-D-N-N-N-N-V-S-G-N-D-N-N-V-S-G-S-F-H-T-V-
S-G-G-H-N-T-V-S-G-S-N-N-T-V-S-G-S-N-H-V-V-S-G-S-N-K-V-V-T-D-A

as well as modified versions thereof.